

## PTHrP regulation and calcium balance in sea bream (*Sparus auratus* L.) under calcium constraint

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### Summary

Juvenile gilthead sea bream were exposed to diluted seawater (2.5‰ salinity; DSW) for 3 h or, in a second experiment, acclimated to DSW and fed a control or calcium-deficient diet for 30 days. Branchial  $\text{Ca}^{2+}$  influx, drinking rate and plasma calcium levels were assessed. Sea bream plasma parathyroid hormone related protein (sPTHrP) was measured, and mRNAs of *pthrp*, its main receptor, *pth1r*, and the calcium-sensing receptor (*casr*) were quantified in osmoregulatory tissues and the pituitary gland. When calcium is limited in water or diet, sea bream maintain calcium balance; however, both plasma  $\text{Ca}^{2+}$  and plasma sPTHrP concentrations were lower when calcium was restricted in both water and diet. Positive correlations between plasma sPTHrP and plasma  $\text{Ca}^{2+}$  ( $R^2=0.30$ ,  $N=39$ ,  $P<0.05$ ), and plasma sPTHrP and body mass of the fish ( $R^2=0.37$ ,  $N=148$ ,  $P<0.001$ ) were found. Immunoreactive sPTHrP was demonstrated in pituitary gland pars intermedia cells that border the pars

nervosa and co-localises with somatolactin. In the pituitary gland, *pthrp*, *pth1r* and *casr* mRNAs were downregulated after both short- and long-term exposure to DSW. A correlation between pituitary gland *pthrp* mRNA expression and plasma  $\text{Ca}^{2+}$  ( $R^2=0.71$ ,  $N=7$ ,  $P<0.01$ ) was observed. In gill tissue, *pthrp* and *pth1r* mRNAs were significantly upregulated after 30 days exposure to DSW, whereas no effect was found for *casr* mRNA expression. We conclude that in water of low salinity, declining pituitary gland *pthrp* mRNA expression accompanied by constant plasma sPTHrP levels points to a reduced sPTHrP turnover and that sPTHrP, through paracrine interaction, is involved in the regulation of branchial calcium handling, independently of endocrine pituitary gland sPTHrP.

Key words: PTHrP, calcium balance, pituitary gland, hypocalcemia, *Sparus auratus*.

### Introduction

Parathyroid hormone related protein (PTHrP) is a hypercalcemic factor in fish (Guerreiro et al., 2001). Phylogenetically it is the ancestor of parathyroid hormone (PTH), which is the major hypercalcemic hormone in terrestrial vertebrates. A sea bream (*Sparus auratus* L.) *pthrp* cDNA has been cloned (Flanagan et al., 2000) and the genomic structure of *pthrp* was clarified in fugu [*Fugu rubripes* Temminck and Schlegel, 1850 (Power et al., 2000)]. Recently, *pth* genes were discovered in the zebrafish (*Danio rerio* Hamilton 1822) genome (Gensure et al., 2004). The two hormones share a high N-terminal amino acid sequence homology and both peptides bind and activate shared G-coupled PTH/PTHrP receptors (Gardella and Jüppner, 2001). Three different PTHrP receptors were identified in fish (PTH1R, PTH2R and PTH3R), of which PTH1R is the most common and shares homology with the mammalian PTH1R (Rubin and Jüppner, 1999). PTHrP has a

key function in several physiological and biochemical processes in fish, including tissue differentiation and proliferation, vitellogenesis (Guerreiro et al., 2002; Bevelander et al., 2006), cortisol production (Rotllant et al., 2005a), calcium regulation (Guerreiro et al., 2001; Abbink et al., 2004) and calcium resorption from bone and scales (Rotllant et al., 2005b), which strongly indicates that PTHrP is involved in (skeletal) calcium physiology. The presence of PTHrP in a large number of tissues suggests PTHrP to be an auto-/intra- or paracrine factor. However, the immunohistochemical detection of PTHrP in the pituitary gland could also suggest a classical endocrine function for PTHrP in fish, as suggested by Danks et al. (Danks et al., 1993).

Fish have access to infinite sources of readily available calcium in the water. Calcium from water and diet can be taken up *via* gills and intestine, and calcium balance is achieved by branchial efflux and intestinal excretion. About 99% of the total

calcium in fish is incorporated in the skeleton and dermal scales (Flik et al., 1986); the latter have a protective function, but also serve as an internal calcium buffer. In fish blood, the plasma total calcium concentration is about 2–3 mmol l<sup>-1</sup>, of which the ionic fraction accounts for about half (Hanssen et al., 1991). This ionic fraction is important for numerous physiological and biochemical processes and is therefore tightly regulated within narrow limits by calcemic endocrines (Flik et al., 1995). As the calcium availability in water and diet vary, as does the need for calcium, the calcemic endocrine system should react swiftly to changes in calcium availability or need (Björnsson et al., 1999).

This study focused on the regulation of the hypercalcemic sPTHrP and the calcium balance in response to a short and long-term calcium constraint in water and/or diet. Juvenile sea bream were rapidly transferred from full-strength seawater (SW; 34‰ salinity; 10.5 mmol l<sup>-1</sup> Ca<sup>2+</sup>) to diluted seawater (DSW; 2.5‰ salinity; 0.7 mmol l<sup>-1</sup> Ca<sup>2+</sup>) and sampled 3 h later (the *short-term experiment*). In a second experiment, juvenile sea bream were exposed to SW or DSW and were fed a calcium-sufficient (Ca+) or calcium-deficient (Ca-) diet for 30 days (the *long-term experiment*). The experiments were carried out under controlled laboratory conditions. Gill Ca<sup>2+</sup> influx ( $F_{in}^{Ca^{2+}}$ ), drinking rate (DR), plasma sPTHrP, as well as plasma total and ionic calcium concentrations were assessed. *pthrp*, *pth1r* and *casr* mRNA expression levels were quantified in gill, intestine, kidney and the pituitary gland and immunostaining was used to examine pituitary glands for sPTHrP immunoreactivity.

## Materials and methods

### Fish

Juvenile gilthead sea bream (*Sparus auratus* L.) were obtained from a commercial fish farm (Viveiro Vilanova, Lda., V.N. Milfontes, Portugal) and flown to the Netherlands without mortality. The fish were kept in round 600 l tanks with aerated flow through, a salinity of 34‰ and a temperature of 23±1°C. The fish were fed commercial pellets (Trouvit, Trouw, Putten, The Netherlands) at a ration of 2% of the total body mass daily.

### Experimental set-up

#### Short-term experiment

Fourteen fish were placed in a tank with identical water conditions as in the stock. After 1 week of acclimatisation, the fish were rapidly transferred to a second tank with identical conditions (SW; control transfer;  $N=7$ ) or to a tank containing diluted seawater of 2.5‰ salinity (DSW;  $N=7$ ). After 3 h, the fish were killed with 2-phenoxyethanol (1:200; Sigma-Aldrich, St Louis, MO, USA) and blood was taken from the caudal vessels using a 1 ml tuberculin syringe, rinsed with 5× diluted sodium heparin (Leo Pharma, Weesp, The Netherlands; 1000 units ml<sup>-1</sup>). The collected blood was centrifuged at 13 600 g for 10 min and the plasma so obtained stored at -20°C. Fish were not fed for 24 h before sampling.

#### Long-term experiment

Fish ( $N=160$ ) were randomly selected from stock, placed in

four round tanks with 40 fish per tank and left to acclimatise. After 1 week, the salinity was gradually lowered by continuous flow-through with demineralised water until the test salinity of 2.5‰ (0.7 mmol l<sup>-1</sup> Ca<sup>2+</sup>) was reached, after 48 h. The diet was changed from control pellets to the test pellets (Hope Farms, Woerden, The Netherlands). The experimental animals were fed first and the controls received an equivalent amount of food as taken up by the experimental fish. After 3 days, the fish fully accepted the new diet and ate all the food provided (2% of the total body mass per day). The four groups of fish included a control (group a: 34‰ salinity; Ca+ diet) and three test groups: group b (34‰ salinity; Ca- diet), group c (2.5‰ salinity; Ca+ diet) and group d (2.5‰ salinity; Ca- diet). After 30 days, the fish were sampled as described for the short-term experiment; on the day before sampling, feeding was discontinued.

### Calcium influx and drinking

After 30 days into the experiment, 20 fish from each group were randomly selected and placed in two identical vessels. After 24 h of acclimatisation, <sup>51</sup>Cr-EDTA (1.9 kBq ml<sup>-1</sup>) or <sup>45</sup>CaCl<sub>2</sub> (2.5 kBq ml<sup>-1</sup>) was added to the tanks to assess drinking rate (DR) and gill Ca<sup>2+</sup> influx ( $F_{in}^{Ca^{2+}}$ ), respectively (Flik et al., 1985). Fish were sampled 2 h (DR) or 4 h ( $F_{in}^{Ca^{2+}}$ ) after addition of the isotopes. Water samples were collected and the fish were killed by adding 2-phenoxyethanol (1:200; Sigma-Aldrich) to the water. The fish were rinsed with demineralised water, quick-frozen in solid CO<sub>2</sub> and the frozen intestinal track was removed. Samples were weighed and rapidly digested in H<sub>2</sub>O<sub>2</sub> (35%; 2 ml g<sup>-1</sup>; Lamers & Pleuger, 's Hertogenbosch, The Netherlands). Water calcium content was measured with a calcium kit (Roche, Mannheim, Germany; cat. no. 1489216) and radioactivity in the water and digested fish samples was counted with a liquid scintillation counter (Wallac 1410; Wallac, Turku, Finland). An OptiPhase HiSafe 3 liquid scintillation cocktail (Perkin-Elmer, Boston, MA, USA) was added before counting.

DR was calculated as:  $DR=A_i/(A_w t m)$ , where  $A_i$  is the total activity of <sup>51</sup>Cr-EDTA in the intestinal track (c.p.m.),  $A_w$  is the total activity in the water (c.p.m. ml<sup>-1</sup>),  $t$  is the exposure time to <sup>51</sup>Cr-EDTA (h) and  $m$  is the mass (mg) of the fish (Flik et al., 2002).

$F_{in}^{Ca^{2+}}$  was calculated as:  $F_{in}^{Ca^{2+}}=(A_f C_w)/(A_w t)$ , where  $A_f$  is the total activity of <sup>45</sup>Ca<sup>2+</sup> in the fish (d.p.m.),  $C_w$  is the calcium concentration in the water (pmol l<sup>-1</sup>),  $A_w$  is the total activity in the water (d.p.m. l<sup>-1</sup>), and  $t$  is the duration of exposure to <sup>45</sup>Ca<sup>2+</sup> (h). There were no differences in total mass between groups, therefore the results are expressed as nl h<sup>-1</sup> (DR) and nmol h<sup>-1</sup> ( $F_{in}^{Ca^{2+}}$ ) and were not normalised for body mass (Guerreiro et al., 2004).

### Plasma analyses

Plasma Ca<sup>2+</sup> (mmol l<sup>-1</sup>) was measured using a Stat Profile pHox plus analyser (Nova Biomedical, Waltham, MA, USA). Plasma total calcium (mmol l<sup>-1</sup>) was assessed using a calcium kit (Roche, Mannheim, Germany) and plasma PTHrP (nmol l<sup>-1</sup>) was measured with a homologous radioimmunoassay according to the method of Rotllant et al. (Rotllant et al., 2003).

### Immunohistochemistry

Juvenile sea bream pituitary glands were fixed in Bouin's fixative for 90 min, dehydrated and embedded in paraffin wax. Sections were cut at 5  $\mu$ m and dewaxed using xylene and degraded alcohols. The immunostaining procedure followed the protocols described earlier for PTHrP (Danks et al., 1993) and somatolactin (SL) (Kaneko et al., 1993). Rabbit anti-sea bream (1-34)sPTHrP (1:100) and rabbit anti-rainbow trout SL (1:3000; a generous gift from Dr Sho Kakizawa, Ocean Research Institute, Tokyo, Japan) were used as primary antibodies. For sPTHrP immunostaining, the sensitive immunoperoxidase method with the Vectastain avidin-biotinylated enzyme complex (Vectastain ABC; Vector Laboratories Inc., Burlingame, CA, USA) was used to increase the staining intensity. Periodic-acid Schiff (PAS) staining was used to distinguish the two cell populations that are found in the pituitary gland, the PAS-positive somatolactin cells in the pars intermedia (pi), and the PAS-negative melanocyte stimulating hormone (MSH) cells.

### Expression of *pthrp*, *pth1r* and *casr* mRNA

Tissue samples from the gill, kidney, anterior part of the intestine and pituitary gland were collected. Real-time, quantitative PCR (qPCR) was used to quantify the mRNA expression levels for *pthrp*, *pth1r* and the *casr* according to Hang et al. (Hang et al., 2005), with the housekeeping gene  $\beta$ -actin as endogenous control.

### Statistics

All data are expressed as means  $\pm$  standard deviation (s.d.); differences among groups were assessed by ANOVA. Significance of differences was assessed by parametric (Student's *t*-test) or non-parametric (Mann-Whitney *U*-test) when appropriate and  $P < 0.05$  was taken as fiducial limit.

## Results

### Gill calcium influx and drinking rate

Fig. 1A shows that branchial  $F_{in}^{Ca^{2+}}$  declined significantly from  $105 \pm 47$  and  $77 \pm 17$  nmol h<sup>-1</sup> in the SW groups a and b to  $21.4 \pm 3.7$  and  $18.0 \pm 4.8$  nmol h<sup>-1</sup> in the DSW groups c and d.

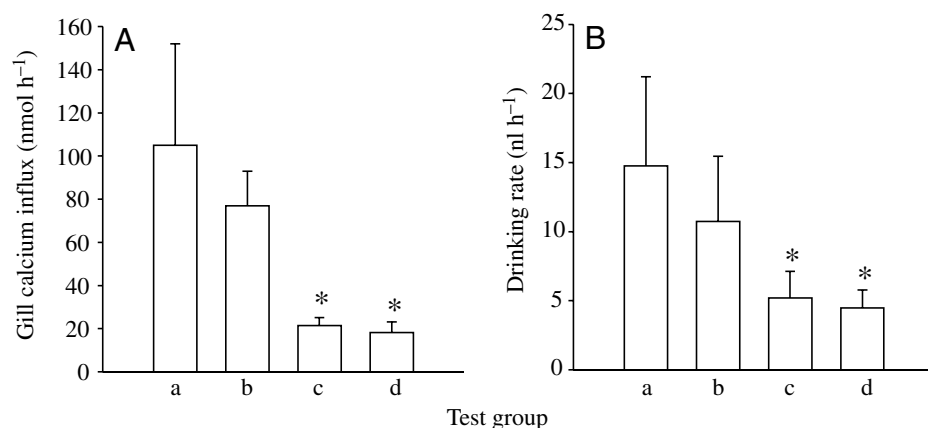


Fig. 1. (A) Gill calcium influx ( $F_{in}^{Ca^{2+}}$ ;  $N=10$ ) and (B) drinking rate ( $DR$ ;  $N=10$ ) significantly decreased in fish exposed to diluted seawater (DSW) for 30 days. The four groups of fish are: group a, control (34‰ salinity, Ca+ diet) and three test groups: group b (34‰ salinity, Ca- diet), group c (2.5‰ salinity, Ca+ diet) and group d (2.5‰ salinity, Ca- diet). Asterisks indicate significant difference ( $P < 0.01$ ) compared with control values. Values are means  $\pm$  s.d.

This same pattern was found for drinking (Fig. 1B), with a significant decrease from  $14.8 \pm 6.5$  and  $10.7 \pm 4.7$  nl h<sup>-1</sup> in SW to  $5.2 \pm 1.93$  and  $4.5 \pm 1.3$  nl h<sup>-1</sup> in the DSW groups. Modification of calcium in the diet had no effect on gill  $F_{in}^{Ca^{2+}}$  or  $DR$ .

### Plasma analyses

Exposure to DSW for 3 h had no effect on the total and ionic plasma calcium level (Fig. 2A). When calcium was limited in water and/or diet for 30 days, the plasma total calcium concentration decreased in all experimental groups, whereas the ionic fraction decreased only when calcium was limited in both water and diet ( $1.13 \pm 0.05$  mmol l<sup>-1</sup> Ca<sup>2+</sup> in group a and  $0.93 \pm 0.07$  mmol l<sup>-1</sup> Ca<sup>2+</sup> in group d).

The plasma sPTHrP level was not affected after exposure to DSW for 3 h (Fig. 2B), with  $0.28 \pm 0.06$  nmol l<sup>-1</sup> in the controls and  $0.26 \pm 0.12$  nmol l<sup>-1</sup> sPTHrP in the DSW fish. In the long-term experiment, the plasma sPTHrP level had slightly, but significantly, decreased when calcium was limited in both water and diet, from  $0.13 \pm 0.02$  nmol l<sup>-1</sup> in the controls (group a) to  $0.10 \pm 0.04$  nmol l<sup>-1</sup> in group d (Fig. 2B). The fish from the short-term experiment were transferred to another tank 3 h before sampling, which could have induced a stress response and thus increased cortisol levels. The measured cortisol levels,  $42.1 \pm 10.1$  nmol l<sup>-1</sup> ( $N=7$ ) in the SW fish and  $38.5 \pm 10.8$  nmol l<sup>-1</sup> ( $N=7$ ) in the DSW fish, do not represent stress levels in sea bream (Arends et al., 1999). We thus exclude stress-induced elevated cortisol levels as a cause of the difference seen in sPTHrP levels between the short and long-term experiment. These differences may relate to different body masses of the groups in the short and long-term experiment.

Fig. 3 shows the correlation that was found between plasma Ca<sup>2+</sup> and plasma sPTHrP (Fig. 3A;  $R^2=0.30$ ,  $N=39$ ,  $P < 0.05$ ) and between plasma sPTHrP and the wet mass of the fish (Fig. 3B;  $R^2=0.37$ ,  $N=148$ ,  $P < 0.001$ ) for all the control observations made.

### Immunohistochemistry

Immunostaining with antisera to sea bream (1-34)sPTHrP and trout (*Oncorhynchus mykiss* Walbaum, 1792) SL revealed

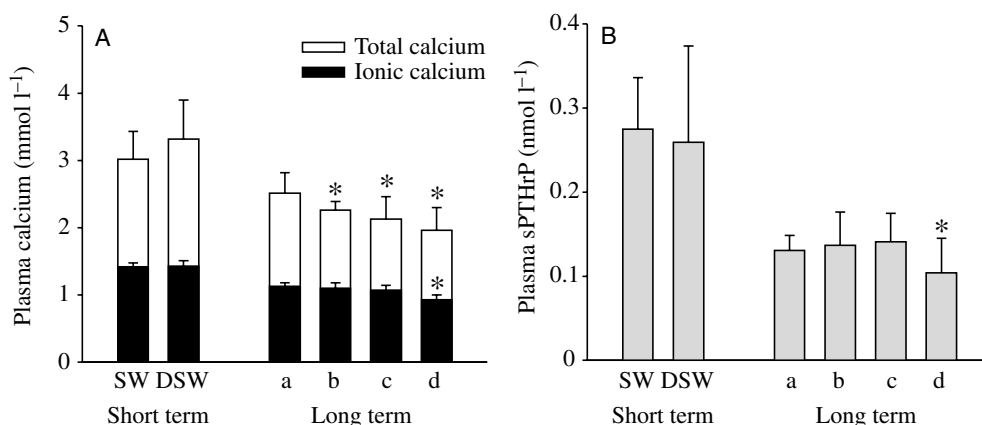


Fig. 2. (A) Plasma calcium levels ( $N=7$  for the short-term experiment and  $N=10$  for the long-term experiment) show the strict control of the ionic fraction, which only slightly, but significantly decreased after long-term calcium constraint in both water and diet. (B) In accordance, plasma sPTHrP decreased significantly in group d compared to controls. Asterisks indicate significant difference ( $P<0.05$ ) from control group a. Values are means  $\pm$  s.d. SW, full strength seawater; DSW, diluted seawater.

immunoreactive sPTHrP and SL in pituitary gland pi cells (Fig. 4). The sPTHrP immunoreactivity was found in the PAS-positive SL cells and bordered the pars nervosa (pn). Controls with omission of the first antibody and pre-absorption with sPTHrP confirmed the specificity of sPTHrP immunoreactivity (data not shown).

#### *Pthrp, pth1r and casr mRNA expression*

Expression of *pthrp*, *pth1r* and *casr* mRNA was significantly downregulated in the pituitary gland of fish exposed to DSW for 3 h (Fig. 5). In kidney, intestine and gill, no effect on *pthrp*, *pth1r* or *casr* mRNA expression was observed (data for kidney and intestine not shown).

Exposure to DSW for 30 days resulted in significant downregulation of *pthrp*, *pth1r* and *casr* mRNAs in the pituitary gland when compared with the control group (Fig. 6A). In gills, a significant upregulation of *pthrp* and *pth1r* mRNA was found in the DSW fish, whereas expression of *casr* mRNA remained constant (Fig. 6B). In intestine and kidney,

no differences in expression of *pthrp*, *pth1r* and *casr* mRNA were found (data not shown).

In the short-term experiment, a strong relationship between plasma  $\text{Ca}^{2+}$  and pituitary gland *pthrp* mRNA expression was found in the controls (Fig. 7;  $R^2=0.71$ ,  $N=7$ ,  $P<0.01$ ), whereas in the DSW group, this relationship was absent. No such samples were available for the long-term experiment.

#### Discussion

This study provides new key findings about the regulatory role of sPTHrP in the calcium balance of juvenile sea bream under calcium constraint.

Branchial  $F_{\text{in}}^{\text{Ca}^{2+}}$  and DR decreased in DSW; plasma  $\text{Ca}^{2+}$  had slightly, but significantly, decreased after long-term exposure to limited calcium in both water and diet. Apparently, DSW induces mild hypocalcemia that is not counteracted by increased  $\text{Ca}^{2+}$  uptake from the water or by drinking.

Plasma sPTHrP level decreased after long-term limitation of

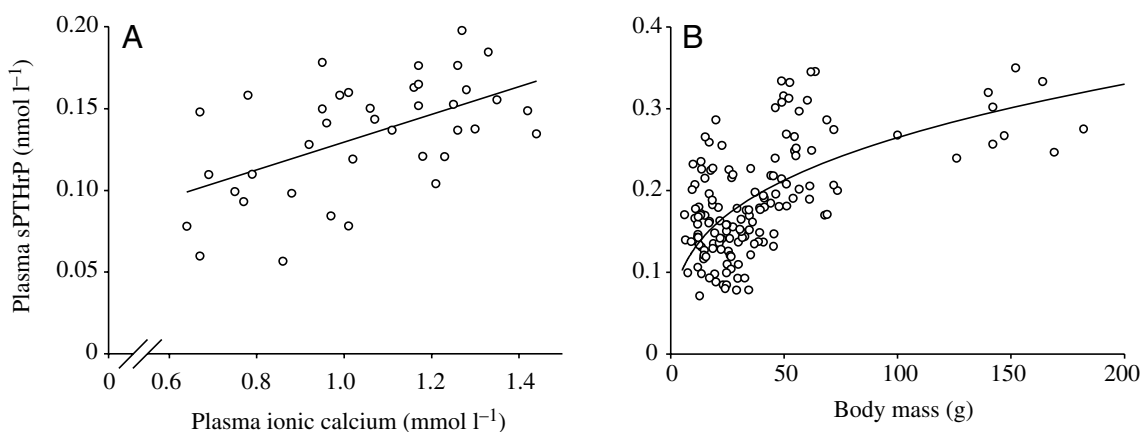


Fig. 3. Positive relationships were found between plasma sPTHrP and (A) plasma ionic calcium (linear regression;  $R^2=0.30$ ,  $N=39$ ,  $P<0.05$ ) and between plasma sPTHrP and (B) the body mass of the fish (power function;  $R^2=0.37$ ,  $N=148$ ,  $P<0.001$ ).



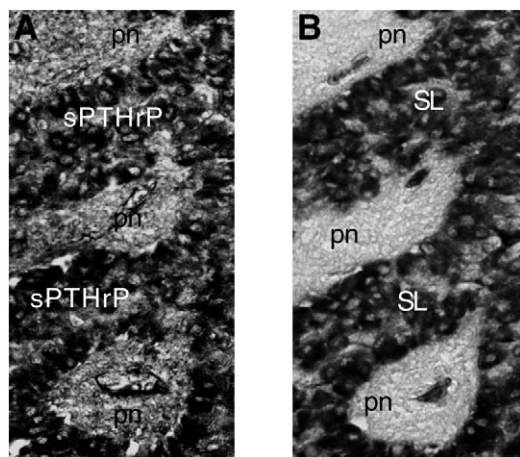


Fig. 4. Immunostaining for (1-34)sPTHrP (A) and somatolactin (SL; B) in sea bream pituitary glands shows the co-localisation for sPTHrP and SL in cells of the pars intermedia, bordering the pars nervosa (pn). Magnification, 400 $\times$ . Controls with omission of the first antibody and pre-absorption with sPTHrP confirmed the specificity of sPTHrP immunoreactivity.

calcium in both water and diet and plasma sPTHrP correlated with plasma  $\text{Ca}^{2+}$  and the body mass of the fish. This points to a decreased calcium turnover under calcium constraint.

Immunoreactive sPTHrP co-localised with immunoreactive SL in PAS-positive cells of the pi in the pituitary gland, indicating that pituitary sPTHrP may be the source of the high plasma sPTHrP levels in the fish.

In the pituitary gland, downregulation of *pthrp*, *pth1r* and *casr* mRNA was observed after both 3 h and 30 days of calcium constraint. In gills, mRNA for *pthrp* and *pth1r* was upregulated, but only after 30 days, whereas *casr* mRNA expression was not

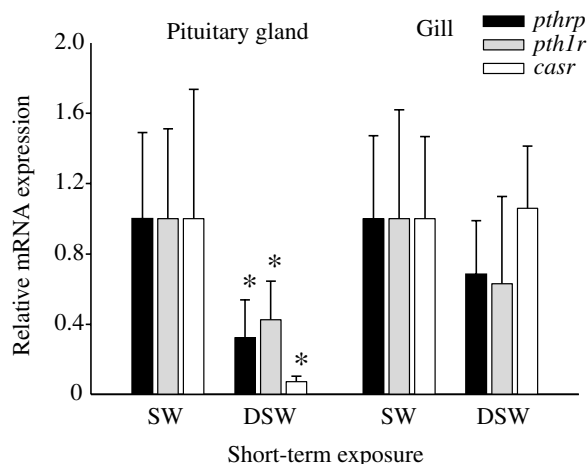


Fig. 5. Expression of *pthrp*, *pth1r* and *casr* mRNA ( $N=7$ ) in the pituitary gland is downregulated in fish after 3 h of exposure to diluted seawater (DSW), whereas no effect was found in gill tissue. Asterisks indicate significant difference ( $P<0.05$ ) compared with full strength seawater (SW) values. Values are means  $\pm$  s.d.

affected by calcium restraint. Thus we have evidence for a branchial sPTHrP regulatory system, acting independently of endocrine pituitary gland sPTHrP actions. The branchial chloride cell, being a key factor in calcium uptake in fish (Flik et al., 1995), appears to be fitted with a para-/auto- or intracrine hypercalcemic hormonal control mechanism. The production of sPTHrP by the chloride cell may be adjusted *via* CaSR activity. *In situ* hybridisation experiments are required for confirmation.

#### Gill $F_{in}^{\text{Ca}^{2+}}$ and DR

Seawater is a strongly hypercalcemic environment for fish ( $\sim 10 \text{ mmol l}^{-1} \text{ Ca}^{2+}$ ) and therefore, fish are forced to reduce  $\text{Ca}^{2+}$  influx or to actively secrete  $\text{Ca}^{2+}$  to compensate for excessive  $\text{Ca}^{2+}$  influx. To compensate for osmotic water loss, seawater fish constantly drink water, which at the same time represents a high  $\text{Ca}^{2+}$  load. The transepithelial potential in fish is always more positive than the equilibrium potential for  $\text{Ca}^{2+}$  across the integument and therefore, the electrochemical gradient for  $\text{Ca}^{2+}$  (the driving force for passive  $\text{Ca}^{2+}$  movement across the gills), is directed outwards (+30 mV), causing a substantial passive  $\text{Ca}^{2+}$  efflux over the leaky branchial epithelium (Flik and Verboost, 1993). The uptake of  $\text{Ca}^{2+}$  is therefore not by diffusion, but by active transport.

The DSW ( $0.7 \text{ mmol l}^{-1} \text{ Ca}^{2+}$ ) causes a decrease in osmotic exchange, which subsequently results in the measured threefold decline in DR (and a consequent decrease in  $\text{Ca}^{2+}$  intake through drinking) and a fivefold decline in gill  $F_{in}^{\text{Ca}^{2+}}$ . In a hypotonic solution such as DSW, the osmotic water loss reverses to water gain and the influx of  $\text{Ca}^{2+}$  occurs almost entirely *via* the gills (Flik et al., 1986). The 15 times lower ambient  $\text{Ca}^{2+}$  concentration combined with a just five fold decline in  $F_{in}^{\text{Ca}^{2+}}$  points to an increase in  $\text{Ca}^{2+}$  influx capacity or efficiency. This could be achieved by an increase in chloride cell density in the branchial epithelium (Flik et al., 1986) and an enhanced prolactin secretion. Low salinity is known to increase prolactin secretion from the pituitary gland (Kaneko and Hirano, 1993) in salt water fishes. Prolactin is known to limit ionic losses and water permeability in osmoregulatory tissues in hyposmotic media and to stimulate  $\text{Ca}^{2+}$  influx through gills and  $\text{Ca}^{2+}$ -ATPase activity in gill plasma membranes (Flik et al., 1994), thereby increasing the  $\text{Ca}^{2+}$  influx capacity. The hypercalcemic control by sPTHrP which is shown in this study may connect both factors. This is further strengthened by the observation that gene expression for *pthrp* in mammals is upregulated in response to increased plasma prolactin levels (Thiede, 1989).

#### Endocrinology

An interesting observation is the relationship between the body mass of the fish and the plasma sPTHrP concentration. In sea bream, the plasma sPTHrP level increases with the body mass and plateaus with increasing mass of the fish, which suggests a decreasing need for hypercalcemic control with increasing body mass. Apparently, hypercalcemic control in juvenile stages is critically dependent on sPTHrP. As the

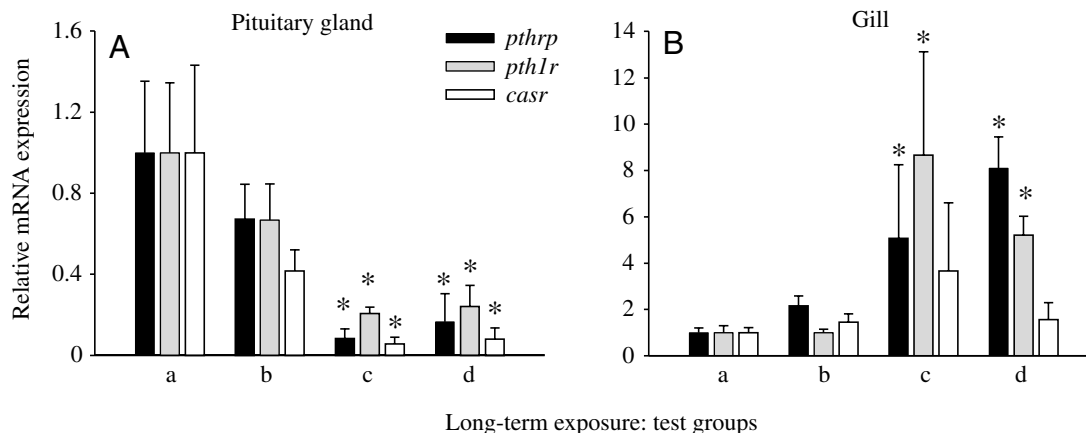


Fig. 6. Expression of *pthrp*, *pth1r* and *casr* mRNAs ( $N=10$ ) is significantly downregulated in pituitary gland (A) in the fish exposed to low calcium in dilute seawater for 30 days, whereas gill tissue (B) shows a significant upregulation of *pthrp* and *pth1r* mRNAs under these conditions. Asterisks indicate significant difference ( $P < 0.05$ ) compared with the controls (group a). Values are means  $\pm$  s.d.

growth rate of fish decreases with age, the need for calcium to be incorporated into the skeleton and scales also decreases, as may the requirement for regulatory sPTHrP. Strong positive correlations between plasma sPTHrP and the whole body content of calcium, phosphorus and magnesium (the main minerals in bone) were found (W. Abbink, X. Hang, P. M. Guerreiro, T. Spanings, A. V. M. Canario and G. Flik, manuscript in preparation), which strengthens the assumption that sPTHrP is involved in skeletal calcium physiology. This was recently suggested by Redruello et al. (Redruello et al., 2005), who showed a downregulation of the unique extracellular calcium binding glycoprotein osteonectin by PTHrP, and by Rotllant et al. (Rotllant et al., 2005b), who reported that PTHrP induced osteoclastic activity in scale tissue, as indicated by its stimulation of tartrate-resistant acid phosphatase (TRAPC; a marker for osteoclastic activity in fish scales).

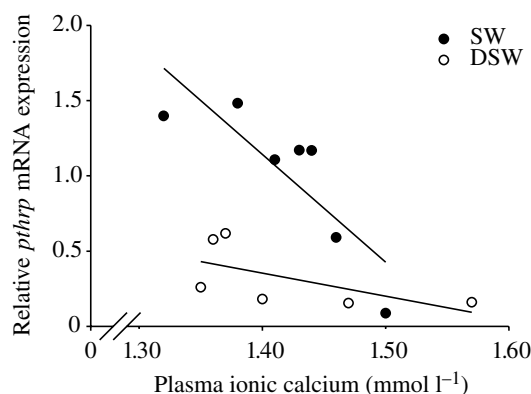


Fig. 7. In the short-term experiment, a negative correlation between pituitary gland *pthrp* expression and plasma  $\text{Ca}^{2+}$  was found in fish exposed to full strength seawater (SW) ( $R^2=0.71$ ;  $N=7$ ;  $P < 0.01$ ). In fish exposed to diluted seawater (DSW), no significant relationship was found. No such data were available for the long-term experiment.

### Immunohistochemistry

In the sea bream pituitary gland, sPTHrP staining was detected in cells of the pi that were near the pn, and these sPTHrP-positive cells were identified as SL-producing cells. This was confirmed in earlier studies, when Rand-Weaver et al. (Rand-Weaver et al., 1991) and Kaneko et al. (Kaneko et al., 1993) found SL staining in PAS-positive cells that border the pn, in several teleosts. Our data confirm an earlier claim by Ingleton et al. (Ingleton et al., 1998) who reported that in sea bream, sPTHrP and SL are both located in the PAS-positive cells and that some cells contained both sPTHrP and SL. SL is a hormone from the *prl* gene family and is structurally related to both PRL and growth hormone. Kakizawa et al. (Kakizawa et al., 1993) studied SL plasma levels and *sl* mRNA expression in rainbow trout and suggested a role for SL in calcium balance and an increased hormone turnover rate at low calcium levels. Changes in SL plasma levels and pituitary gland mRNA expression at low ambient calcium appear only after several days (Kakizawa et al., 1993), which makes short-term effects of SL on calcium balance unlikely. However, the activity of SL-producing cells may be affected indirectly, possibly by the action of PTHrP. Our data show sPTHrP immunostaining in sea bream pituitary gland SL-producing cells and activation of *pthrp* mRNA production in the pituitary gland 3 h after transfer from SW to DSW. It could very well be that, in cells co-expressing *pthrp* and *sl*, the *pthrp* upregulation precedes that of *sl* and therefore the activity of SL in the pituitary gland. Interestingly, the pituitary gland sPTHrP-producing cells colocalise with a sub-population of SL-producing cells, the  $\text{SL}\alpha$  cells as observed in zebrafish (Zhu et al., 2004).

### mRNA expression

Expression of *pthrp* and *pth1r* mRNA was found in all tissues examined, indicative of an auto-/para- or intracrine function of sPTHrP. However, circulating plasma PTHrP levels in teleosts and elasmobranchs, as well as immunostaining (Trivett et al., 1999) and mRNA expression (Hang et al., 2005)

for *pthrp* in pituitary glands have been established, pointing to an endocrine function for PTHrP as well.

Downregulation of *pthrp* and *pth1r* mRNA in the pituitary gland was established after 3 h of calcium constraint and remained reduced after at least 30 days of calcium constraint. Both a rapid activation of pituitary gland sPTHrP production and a long-term involvement of sPTHrP in the adaptation to hypocalcemic media seem required to maintain calcium balance at hypocalcemic conditions. This suggestion is supported by the correlations that were found between plasma  $\text{Ca}^{2+}$  and pituitary gland *pthrp* mRNA expression and between plasma  $\text{Ca}^{2+}$  and plasma sPTHrP protein. However, the downregulation of *pthrp* and *pth1r* mRNA in the pituitary gland was not accompanied by a change in plasma sPTHrP levels, which had only slightly decreased in the group that was held in DSW and fed a Ca-diet for 30 days. The adaptive response to calcium constraint results in a reduced metabolic clearance of sPTHrP from the plasma (in contrast to the reported action of SL), with downregulated mRNA expression in the pituitary gland and unaltered plasma sPTHrP levels. This points to a differential regulation of release of sPTHrP and SL in the cells that co-express these proteins, or alternatively a differential regulation of the two pituitary SL cell populations, recently reported (Zhu et al., 2004).

The five- to eightfold upregulated peripheral *pthrp* and *pth1r* mRNA levels that were found in gills after 30 days exposure to low calcium may reflect an adaptive response, possibly as a result of a decreased environmental calcium concentration or the reduced sPTHrP metabolic clearance from the plasma, by an autoregulatory feedback of sPTHrP on its own secretion. In earlier studies on mammals, Fujimi et al. (Fujimi et al., 1991) suggested that PTH(1-34) directly inhibits PTH secretion in parathyroid cells. In contrast, Lewin et al. (Lewin et al., 2003) hypothesized that PTH has a positive auto-feedback on its own secretion under hypocalcemic conditions in rats.

Flanagan et al. (Flanagan et al., 2000) showed PTHrP staining in the chloride cells of gills in teleosts and identified these cells as the principal location of PTHrP in gill tissue. The increase in chloride cell density in diluted seawater conditions could explain the increase in *pthrp* mRNA that was found in gills. Chloride cells are the site of branchial  $\text{Ca}^{2+}$  uptake in gills and they also contain CaSR; however, expression of *casr* mRNA was unaffected in gills after calcium restraint. CaSR is regulated and equipped to respond to the blood  $\text{Ca}^{2+}$  level. Fluctuations as small as  $0.2 \text{ mmol l}^{-1}$  are sensed (Lopez-Illasaca et al., 1997), which enables the fish to tightly regulate the blood  $\text{Ca}^{2+}$  concentration. In the present study, the plasma  $\text{Ca}^{2+}$  level was remarkably constant and maintained within a maximal range of  $0.2 \text{ mmol l}^{-1}$  difference when compared with the controls and therefore upregulation of *casr* mRNA may not be relevant. In the pituitary gland, a significant downregulation of *casr* mRNA was observed after both short- and long-term calcium constraint, indicating that the need for calcium controlled processes had decreased or reflecting a need for desensitisation to  $\text{Ca}^{2+}$  signals. Flanagan et al. (Flanagan et al., 2002) located immunostaining for CaSR in cells bordering the

pn in both the pi and the pars distalis in the sea bream pituitary gland and suggested a possible feedback between nerve axons from hypothalamic nuclei and pituitary factors affected by calcium. This is supported by the localisation of sPTHrP in cells near the pn in this study, thereby possibly controlling the upregulation of *pthrp* gene expression in the gills.

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